Role of the CaN-NFATc3 Pathway in Restenosis after Rat Abdominal Aorta Balloon Angioplasty Postprint

Authors: Li Xiaoyun, Xiong Longgen, Li Deen, Zhao Luning, Dong Qi

Date: 2017-12-21T00:00:00+00:00

Abstract

Objective: To investigate the role of calcineurin (CaN) and its downstream nuclear factor of activated T cells (NFATc3) in restenosis after balloon angioplasty, and to provide a new theoretical basis for the prevention and treatment of vascular restenosis. Methods: Male SD rats were randomly divided into a sham operation group (n=12), a balloon group (n=12), and a cyclosporine (CsA) group (n=12). Rats in the balloon group underwent balloon angioplasty to injure the abdominal aorta; rats in the CsA group were administered CsA 12.5 mg/(kg · d) by gavage daily from 3 days before surgery until the end of the experiment. Samples were collected 30 days after balloon injury. Vascular tissues were subjected to hematoxylin-eosin (HE) staining and immunohistochemical detection of CaN levels in the vascular wall, with pathological changes observed under an optical microscope; real-time PCR technology was used to detect mRNA expression of CaN and NFATc3 in vascular wall tissues; ELISA was used to determine serum MCP-1 levels. Results: After balloon injury, neointima formation occurred in the vascular wall with uneven thickness; compared with the balloon injury group, intimal hyperplasia and intima/media thickness were significantly reduced in the CsA group ($P<0.05$). Compared with the sham operation group, CaN protein and mRNA expression in vascular wall tissues were significantly increased, NFATc3 mRNA expression was markedly elevated, and plasma inflammatory factor MCP-1 level was also increased in the balloon injury group ($P<0.05$). All the above indices in the CsA group were significantly lower than those in the balloon injury group ($P<0.05$). Conclusion: The CaN-NFATc3 pathway is involved in the development of restenosis after balloon injury in rats; CsA attenuates restenosis formation by inhibiting this pathway.

Full Text

Role of CaN-NFATc3 Pathway in Abdominal Aorta Restenosis Following Balloon Dilatation in Rats

LI Xiaoyun[1], XIONG Longgen[1], LI De' en[1], ZHAO Luning[2], DONG Qi[3] [1]Department of Cardiology, Second Affiliated Hospital of Guangzhou Medical University, Guangzhou 510260, China [2]Experimental Medical Research Center, [3]Department of Physiology, Guangzhou Medical University, Guangzhou 511436, China

Abstract

Objective: To investigate the role of calcineurin (CaN) and its downstream nuclear factor of activated T-cells (NFATc3) in abdominal aorta restenosis following balloon dilatation in rats, providing a novel theoretical basis for preventing and treating vascular restenosis.

Methods: Male SD rats were randomly divided into sham-operated group (n=12), balloon injury group (n=12), and cyclosporine A (CsA) group (n=12). Rats in the latter two groups underwent abdominal aorta injury with balloon dilatation, while those in the CsA group were treated with CsA at a daily dose of 12.5 mg/kg from 3 days before surgery until the end of the experiment. Thirty days after injury, histological analysis of the arterial wall was performed with HE staining and immunohistochemistry. The expressions of CaN and NFATc3 in the abdominal aortas were detected with real-time PCR, and serum concentration of MCP-1 was determined using enzyme-linked immunosorbent assay.

Results: Intimal hyperplasia with irregular thickness of the neointima was observed in the aorta of rats with balloon injury. In rats with CsA treatment, the area of the intimal layers and the ratio of the intimal to the medial layers were significantly lower than those in the balloon injury group ($P<0.05$). Compared to the sham-operated group, the expressions of calcineurin protein and mRNA and NFATc3 mRNA in the arterial wall and serum level of MCP-1 increased significantly in the balloon injury group ($P<0.05$). CsA treatment significantly suppressed aorta restenosis and the alterations of CaN, NFATc3, and serum MCP-1 induced by balloon dilatation ($P<0.05$).

Conclusion: The CaN-NFATc3 signal transduction pathway mediates restenosis of rat abdominal aorta following balloon dilatation, and CsA can attenuate restenosis by suppressing this pathway.

Key words: restenosis; calcineurin; NFATc; cyclosporine A

Introduction

Percutaneous coronary intervention (PCI) is currently one of the effective methods for treating ischemic heart disease caused by coronary atherosclerosis. However, due to factors such as plaque rupture, endothelial denudation, and medial injury caused by the procedure, 30%-50% of patients may develop restenosis at the surgical site within 3-6 months postoperatively. The underlying mechanisms remain unclear, and therapeutic options are limited.

The Ca^2 /CaM-CaN-NFATc signaling system is a key pathway regulating immune system gene transcription. Recent studies have shown that this pathway also exists in nerve, skeletal, cardiac, and smooth muscle cells, participating in various physiological and pathological processes. While its role in cardiac hypertrophy has been extensively studied, research on its involvement in restenosis remains limited. The NFAT family consists of five isoforms that promote vascular smooth muscle cell (VSMC) migration and proliferation. Blocking this signal can reduce neointima formation in injured arteries, but the specific role of each isoform remains unclear. NFATc3 participates in hypoxia-induced pulmonary artery remodeling and regulates endothelial migration and angiogenesis through COX2, yet whether it is involved in post-PCI vascular restenosis is unknown.

Vascular wall inflammatory responses play a critical role in PCI restenosis. Monocyte infiltration into the vascular wall commonly occurs in early lesions, mediated by chemokines such as MCP-1, which induce monocyte migration and participate in vascular disease formation. Blocking MCP-1 can prevent neointima formation after carotid artery balloon dilation. The relationship between the CaN-NFATc pathway and MCP-1 in restenosis formation has not yet been elucidated.

This study employed a rat abdominal aorta balloon injury restenosis model to observe changes in CaN, NFATc3, and the inflammatory factor MCP-1, preliminarily exploring the relationship between the CaN-NFATc pathway and MCP-1, and investigating the intervention effect of the relevant inhibitor CsA on vascular restenosis.

Materials and Methods

1.1 Major Instruments and Surgical Equipment PTCA balloon catheter (20 mm × 1.5 mm, Abbott Laboratories), ultramicrotome (Leica RM2015, Germany), inverted fluorescence biological microscope (1500X/CC) (Leica, Germany), ABI7500 fluorescence quantitative PCR instrument (ABI, USA), PCR instrument (Bio-rad, USA), refrigerated centrifuge (Model 5415, Eppendorf, Germany), and nucleic acid protein quantifier (Nano Drop, USA).

1.2 Major Reagents Fluorescence quantitative PCR kit (TaKaRa), reverse transcription kit (TaKaRa), Trizol reagent (Invitrogen), PCR primers (Invitro-

gen), rat MCP-1 ELISA kit, and CaN primary antibody (Abcam).

1.3 Experimental Animals and Grouping Thirty-six male SD rats weighing 350-450 g and aged 10-12 weeks (purchased from Guangdong Laboratory Animal Monitoring Institute) were randomly divided into sham-operated group, balloon group, and CsA group, with 12 rats in each group. Rats in the balloon group underwent abdominal aorta balloon dilation; rats in the CsA group were administered CsA 12.5 mg/kg · d from 3 days before surgery until the end of the experiment; rats in the sham-operated group only had the balloon catheter inserted without dilation and received no intervention.

1.4 Animal Model Establishment Following the method of Shi Yongying, rats were anesthetized with 3% pentobarbital sodium solution at 50 mg/kg by intraperitoneal injection. The PTCA balloon catheter (2 mm × 15 mm) was inserted from the left common carotid artery to the end of the abdominal aorta. The balloon was inflated with saline to maintain a pressure of 6 ATM for 30 s, then deflated. After a 60 s interval, the balloon was reinflated with saline to maintain 6 ATM pressure, and this procedure was repeated 3 times before the balloon was withdrawn. The sham-operated group underwent the same procedures except for balloon catheter insertion. Animals were sacrificed 30 days post-surgery for sample collection.

1.5 Routine Pathological Examination Injured vascular segments were collected, paraffin-embedded, sectioned, and stained with hematoxylin-eosin (HE). Optical microscopy and Image-Pro Plus 6.0 image analysis system were used for image analysis of HE-stained sections from each group. The circumferences of the external elastic lamina, internal elastic lamina, and lumen were measured first, then the radius of each cross-section was calculated according to the formula circumference = 2 r. Vascular neointimal thickness and medial thickness were then calculated stepwise, where intimal thickness = (internal elastic lamina circumference - lumen circumference)/2 , and medial thickness = (external elastic lamina circumference - internal elastic lamina circumference)/2 . Each slice was measured three times.

1.6 Fluorescence Quantitative PCR Detection of CaN and NFATc3 Expression Total RNA was extracted from samples according to Trizol reagent instructions. Reverse transcription and fluorescence quantitative PCR reactions were performed using TaKaRa reverse transcription kit and fluorescence quantitative PCR kit, respectively. After reaction completion, the amplification and melting curves of Real-Time PCR were confirmed, and CT values were recorded. The relative expression was calculated as follows: ΔΔCt = (Ct target gene - Ct housekeeping gene) experimental group - (Ct target gene - Ct housekeeping gene) control group. In this experiment, the sham-operated group served as the control, and the relative mRNA expression levels in other groups were expressed as 2^(-ΔΔCt) fold of the control group .

Table 1. Primer sequences of CaN, NFATc3 and -actin for real-time PCR

Gene	Upstream Primer	Downstream Primer
-actin	gacaggatgcagaaggagattact	tgatccacatctgctggaaggt
CaN	agtcacagtttgcgggga	agcgagtgttggcaggag
NFATc3	gtgacccagcatcgtttcca	ggctggtgttccaagtcagacata

1.7 ELISA Detection of Plasma Monocyte Chemoattractant Protein-1 (MCP-1) Supernatant prepared from rat venous blood was used to detect MCP-1 levels by ELISA. The test sample (10 L) was added to sample diluent (40 L) and incubated for 30 min, followed by washing and addition of 50 L enzyme-labeled working solution. After incubation for 30 min and plate washing, 50 L each of chromogenic solutions A and B were added sequentially to each well, gently shaken for 30 s, and developed at 37°C for 15 min in the dark. The reaction was terminated with 50 L stop solution, and absorbance values (A values) of each well were measured at 450 nm wavelength using a microplate reader within 15 min. Based on the concentrations and corresponding A values of standard samples, a linear regression equation of the standard curve was calculated, and the corresponding concentration of samples was calculated from their A values. The final concentration was the measured value multiplied by the dilution factor.

1.8 Immunohistochemical Staining Tissue sections (5 m) were routinely deparaffinized and hydrated. After antigen retrieval and blocking, CaN antibody (primary antibody dilution 1:1000, Abcam) was added and incubated overnight. After adding secondary antibody, DAB color development was performed. Yellow or brownish granules in the cytoplasm or nucleus indicated positive signals. Using the Image-Pro Plus 6.0 image analysis system, the average optical density value was measured to represent the intensity of positive products. Higher average values indicated stronger expression intensity of positive reaction products and higher protein content.

1.9 Statistical Analysis SPSS 13.0 statistical software was used to process experimental data. Data were expressed as mean ± standard deviation. One-way ANOVA was performed, followed by pairwise comparison of sample means. $P<0.05$ was considered statistically significant.

Results

2.1 Pathomorphological Examination In the sham-operated group, the vascular wall appeared circular with uniform thickness. Endothelial cells on the luminal side were arranged neatly and symmetrically, with clear and intact

internal and external elastic laminae, and no VSMC infiltration beneath the intima. The balloon group showed reduced lumen area, uneven vessel wall, visible internal elastic lamina rupture, significant neointima formation with irregular thickness, increased extracellular matrix, absence of endothelial cells, and numerous VSMCs near the internal elastic lamina arranged disorderly with local longitudinal smooth muscle arrangement, showing a trend of growing toward the intima. The CsA group showed expanded lumen area compared with the simple model group, with only slight neointima formation beneath the intima [Figure 1: see original paper]. Intimal thickness and intima/media thickness ratio in the balloon injury group and CsA group were significantly higher than in the sham-operated group ($P<0.05$), while intimal thickness and intima/media thickness ratio in the CsA treatment group were significantly lower than in the balloon injury group ($P<0.05$,).

Figure 1. Intimal hyperplasia of the abdominal aorta in each group (Original magnification: ×200). A: Sham group; B: Balloon injury group; C: CsA group.

Table 2. Intimal hyperplasia of rat abdominal aorta induced by balloon injury in each group (Mean±SD)

Group	Thickness of intimal layers (m)	Thickness of medial layers (m)	Ratio of intimal to the medial layers
Sham group	10.004±2.163	311.497±53.460	0.033±0.006
Balloon group	202.275±66.175*	382.310±60.455	0.519±0.103*
CsA group	70.700±21.021*	360.065±97.774	0.200±0.062*

*$P<0.05$ compared with Sham group; $P<0.05$ compared with Balloon group.

2.2 Vascular Wall CaN Expression As shown in [Figure 2: see original paper], the vascular wall in the sham-operated group almost did not express CaN. In the balloon group, brownish-yellow or brown granules appeared in the cytoplasm of most VSMCs in the vascular media and intima, with significantly increased expression compared with the sham-operated group (0.06892±0.01856 vs 0.00712±0.00229, $P<0.05$). Compared with the balloon group, the cyclosporine group showed significantly decreased expression in VSMCs of the media and intima (0.01610±0.00220 vs 0.06892±0.01856, $P<0.05$).

Figure 2. Expression of CaN in abdominal aorta in each group (×400). A: Sham group; B: Balloon injury group; C: CsA group. *$P<0.05$ vs sham-operated group; $P<0.05$ vs balloon injury group.

2.3 Vascular Wall CaN and NFATc3 mRNA Expression Levels Compared with the sham-operated group, vascular tissue CaN and its downstream NFATc3 expression in the balloon group increased significantly (P<0.05), while CaN and NFATc3 expression in the CsA group were significantly lower than in the balloon group (P<0.05) but higher than in the sham-operated group ().

Table 3. Expression of CaN and NFATc3 mRNA in the abdominal aorta of each group (Mean±SD)

Group	CaN	NFATc3
Sham group	1.110±0.608	1.063±0.365
Balloon group	2.143±0.831*	2.076±0.698*
CsA group	1.146±0.336	1.071±0.436

*P<0.05 compared with Sham group; P<0.05 compared with Balloon group.

2.4 Plasma MCP-1 Levels in Rats Plasma MCP-1 level in the balloon group increased by 48% compared with the sham-operated group (813±110 vs 423±66, P<0.05). The CsA group was significantly lower than the balloon group (439±53 vs 813±110, P<0.05) and showed no significant difference from the sham-operated group.

Discussion

PCI restenosis results from multiple factors, including smooth muscle cell proliferation, matrix secretion, and smooth muscle cell migration to the intima at the injury site, leading to excessive intimal thickening and subsequent restenosis. These series of reactions are associated with inflammatory responses, and various growth factors and cytokines released by inflammatory cells at the injury site may be the initiating factors for restenosis lesions. The Ca^2 /CaM-CaN-NFATc signaling system is a key pathway in immune responses with a defined role in inflammatory reactions, and recent studies have further revealed its involvement in cellular growth processes such as hypertrophy and proliferation.

Previous in vitro experimental results showed that NFAT translocation is associated with vascular smooth muscle cell proliferation. After rat aortic VSMCs were stimulated to proliferate by PDGF-BB, significant nuclear translocation of NFATc3 occurred, and both the CaN inhibitor CsA and the NFATc inhibitor INCA-6 effectively reduced the effect of PDGF-BB. The NFATc inhibitor VIVIT could intervene in the pro-proliferative effect of PDGF-BB on VSMCs. AngII (10 -10 mol/L) increased CaN activity in VSMCs in a concentration-dependent manner and promoted cell proliferation, while CsA significantly reduced AngII-induced VSMC proliferation activity and nuclear PCNA expression levels.

Using an in vivo animal model, this study caused arterial restenosis through balloon injury and found that arterial intimal hyperplasia was significantly improved after CsA intervention, with only slight neointima formation beneath the intima and expanded lumen area compared with the simple model group. Haixiang et al. also reported that the NFAT-CaN interaction inhibitor MCV1 could inhibit NFAT activation and reduce restenosis formation after carotid artery denudation. In this experiment, we used only balloon dilation to cause arterial injury to more closely approximate clinical reality, and the results were similar to the denudation method used in most current laboratories, demonstrating that inhibiting the Ca^{2} /CaM-CaN-NFATc signaling system can effectively intervene in vascular restenosis after injury.

This study further found that CaN and NFATc3 mRNA expression levels were significantly elevated in injured vessels. NFATc includes five nuclear transcription factors: NFATc1 (NFAT2/c), NFATc2 (NFAT1/p), NFATc3 (NFAT4/x), NFATc4 (NFAT3), and NFAT5. Whether each isoform participates in restenosis formation and their specific roles remain unclear. In vitro VSMC scratch experiments showed that NFATc1 protein expression increased transiently while accumulating in the nucleus. After bare metal stent implantation in coronary arteries, local vascular intimal hyperplasia occurred with increased NFATc4 expression. The role of NFATc3 in restenosis is currently unclear. Studies have shown that oxidative stress can activate NFATc3, which promotes pulmonary artery smooth muscle cell proliferation and causes vascular remodeling. NFATc3 also promotes COX2 expression and activation in endothelial cells, mediating COX2-dependent endothelial cell migration and proliferation. Some results indirectly suggest a relationship between NFATc3 and restenosis. The chronic inflammatory factor OPN associated with vascular diseases shows increased expression under high glucose stimulation, and inhibiting NFATc3 can reduce OPN overexpression in arteries of diabetic rats. During inflammation, two proteins AIF-1 and IRT-1 are produced, where AIF-1 stimulates VSMC migration and proliferation while IRT-1 has opposite effects, and silencing the NFATc3 gene can reduce the AIF-1/IRT-1 ratio. This study directly confirmed altered NFATc3 expression in a restenosis animal model, suggesting its potential involvement in the pathological process of restenosis.

MCP-1 is an important inflammatory factor secreted by monocytes, macrophages, endothelial cells, and smooth muscle cells, playing a significant role in inflammatory development. It has chemotactic and activating effects on monocytes, macrophages, and basophils, and aggregated monocytes/macrophages can further promote MCP-1 production. When vascular endothelial cells are injured, MCP-1 secretion increases, chemoattracting monocytes/macrophages to the vascular injury site. Monocyte/macrophage infiltration in the neointima of injured arteries can synthesize and secrete numerous inflammatory cytokines and growth factors, stimulating medial VSMCs to migrate and proliferate into the intima and produce large amounts of extracellular matrix, leading to neointima formation and restenosis. Additionally, MCP-1 regulates vascular endothelial cell adhesion molecule expression and

directly induces VSMC migration and proliferation, thereby participating in restenosis formation.

The relationship between the CaN signaling pathway and MCP-1 in restenosis has rarely been reported. Hiroshi et al. found that activated CaN could promote MCP-1 expression in VSMCs, and CsA could inhibit AngII-induced MCP-1 expression. This study found that peripheral blood MCP-1 in rats with simple balloon injury increased significantly and was inhibited by CsA, suggesting that the inflammatory response after vascular injury may be systemic. We speculate that CsA may inhibit inflammatory factors such as MCP-1, thereby preventing monocyte/macrophage aggregation in the neointima of injured arteries, reducing local inflammation in injured arteries, and ultimately inhibiting medial VSMC migration and proliferation. Recent studies have reported on the relationship between NFATc isoforms and MCP-1. Knockout of NFATc1 can block MCP-1-induced activation of the cyclin D1-CDK6-CDK4-Pak1 signaling axis, which mediates VSMC proliferation, migration, and intima formation after vascular injury. The relationship between NFATc3 and MCP-1 remains unclear and warrants further investigation.

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(Edited by: Wu Jinya)

Note: Figure translations are in progress. See original paper for figures.